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ORIGINAL CONTRIBUTION

Compensatory role of the NBCn1 sodium/bicarbonate cotransporter on Ca^{2+}-induced mitochondrial swelling in hypertrophic hearts

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Abstract NBC Na^+/HCO_3^- cotransporter (NBCn1) and NHE1 Na^+/H^+ exchanger have been associated with cardiac disorders and recently located in coronary endothelial cells (CEC) and cardiomyocytes mitochondria, respectively. Mitochondrial NHE1 blockade delays permeability transition pore (MPTP) opening and reduces superoxide levels, two critical events exacerbated in cells of diseased hearts. Conversely, activation of NBCn1 prevented apoptosis in CEC subjected to ischemic stress. We characterized the role of the NHE1 and NBCn1 transporters in heart mitochondria from hypertrophic (SHR) and control (Wistar) rats. Expression of NHE1 was analyzed in left ventricular mitochondrial lysates (LVML) by immunoblots. NHE1 expression increased by ~40% in SHR compared to control ($P < 0.05$, $n = 4$). To examine NHE1-mediated Na^+/H^+ exchange activity in cardiac hypertrophy, mitochondria were loaded with BCECF-AM dye and the maximal rate of pHm change measured after the addition of 50 mM NaCl. SHR mitochondria had greater changes in pHm compared to Wistar, 0.11 ± 0.01 vs. 0.06 ± 0.01, respectively ($P < 0.05$, $n = 5$). In addition, mitochondrial suspensions from SHR and control myocardium were exposed to 200 µM $CaCl_2$ to induce MPTP opening (light-scattering decrease, LSD) and swelling. Surprisingly, SHR rats showed smaller LSD and a reduction in mitochondrial swelling, 67 ± 10% ($n = 15$), compared to control, 139 ± 10% ($n = 13$). NBC inhibition with S0859 (1 µM) significantly increased swelling in both control 139 ± 10% ($n = 8$) and SHR 115 ± 10% ($n = 4$). Finally, NBCn1 Na^+/HCO_3^- cotransporter increased by twofold its expression in SHR LVML, compared to normal ($P < 0.05$, $n = 5$). We conclude that increased NBCn1 activity may play a compensatory role in hypertrophic hearts, protecting mitochondria from Ca^{2+}-induced MPTP opening and swelling.

Keywords Hypertrophy · Myocardium · Mitochondrial permeability transition pore · NBCn1 Na^+/HCO_3^- cotransporter

Introduction

Sarcolemmal Na^+/HCO_3^- cotransporters, electroneutral NBCn1 and electrogenic NBCe1, and Na^+/H^+ exchanger (NHE1) are key intracellular pH (pH_i) regulatory mechanism, major alkaline loaders, and primary Na^+ influx pathways across plasma membrane of cardiac muscle cells. Both NBC and NHE1 have been associated with cardiac disorders [15, 32, 53, 58], and recently located in mitochondria of cardiomyocytes and coronary endothelial cells, respectively [27, 33, 52].

In cardiac muscle cells, mitochondrial dysfunction triggers the causes of frequent cardiac diseases, including ischemia/reperfusion (I/R) injury, decompensated cardiac hypertrophy, and heart failure [38]. The mitochondrial death pathway features the sequential loss of mitochondrial membrane potential ($\Delta\Psi m$), accompanied by irreversible opening of the mitochondrial permeability transition pore (MPTP), release of high levels of reactive oxygen species (ROS), release of toxic proteins into the cytoplasm, and activation of proteolytic caspases activity [12, 50, 55]. The

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pathologic events triggered by I/R orchestrate the opening of the MPTP, which appears to represent a common end-effector of the downward spiral of heart failure [34]. The MPTP is a voltage-sensitive unselective channel, which instigates necrotic cell death or apoptotic cell death (when ATP is somehow maintained) during cardiac disease [34].

The specific NHE1 inhibitor, cariporide, is cardioprotective through its effects on mitochondria [19, 26, 50]. The beneficial effect of NHE1 inhibition by cariporide in hearts subjected to I/R was associated with attenuation of MPTP opening and reduction of apoptosis, reducing mitochondrial dysfunction [26]. In addition, inhibition of mitochondrial NHE during ischemia reduces acidification of the mitochondrial matrix and ATP hydrolysis, delaying the progression of ischemic injury by decreasing cell death [46]. More recently, NHE1 expression and activity were reported in mitochondrial membranes of cardiomyocytes [5]. Inhibition of mitochondrial NHE1 increased the threshold for the opening of the MPTP and subsequent mitochondrial swelling [52] and hallmarks of the mitochondrial death pathway. Moreover, inhibition of NHE1 reduced changes in the altered mitochondrial fission–fusion protein expression affected by both post-infarction remodeling and in vitro cardiomyocyte hypertrophy [27]. Conversely, NBCn1 Na^+/HCO_3^- cotransporter (SLC4A7), which co-localized with mitochondria, modulates the mitochondrial apoptotic pathway during ischemic stress of coronary endothelial cells [33]. Pharmacological inhibition of mitochondrial NBCn1 or targeted knockdown of mitochondrial NBCn1 aggravated ischemia-induced mitochondrial-mediated apoptosis of coronary cells [33].

Recently, abnormal mitochondrial Ca^{2+} handling was demonstrated in hearts from spontaneously hypertensive rats (SHR) [43]. SHR heart mitochondria display reduced Ca^{2+}-induced MPTP opening, increased retention capacity and velocity of mitochondrial Ca^{2+} influx and efflux, and lower $\Delta\Psi m$ compared to control hearts [43].

The aim of the present study was to characterize the role of ion transporters in mitochondria of 18- and 35-week-old hypertrophic SHR heart hearts, and its respective age and sex-matched normal rat heart controls. We report the influence of the mitochondrial NHE1 Na^+/H^+ exchanger and the mitochondrial NBCn1 Na^+/HCO_3^- cotransporter on the Ca^{2+}-induced MPTP opening and mitochondrial swelling as signature of the mitochondrial dysfunction, assessed in the hypertrophic myocardium of SHR rats. Furthermore, we revealed the expression of NBCn1, a distinctive integral plasma membrane protein, in heart mitochondria.

We demonstrated that increased mitochondrial NHE1 expression and function in the myocardium of SHR rats are compensated by enhanced NBCn1 activity, reducing Ca^{2+}-induced mitochondrial swelling and MPTP opening in hypertrophic hearts.

Materials and methods

Isolation of rat cardiomyocytes

Adult rats were anaesthetized with ketamine (100 mg kg^{-1} i.p.) and diazepam (10 mg kg^{-1} i.p.). Animal protocols conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Argentine Republic Law No. 14346 concerning animal protection, and were approved by the Animal Welfare Committee of La Plata School of Medicine (Ref # 4/2015). The hearts were rapidly removed and ventricular myocytes obtained by enzymatic dissociation, using the standard protocols [4].

Isolation of rat heart mitochondria

Rat heart mitochondria were isolated by differential centrifugation, according to a modified method described previously [36]. Briefly, hearts were rapidly excised from ketamine/diazepam-anaesthetized rats and placed in an ice-cold isolation buffer (IB) containing (in mM): 75 sucrose, 225 mannitol, 0.01 EGTA, pH 7.4. After both atria and right ventricle were removed, the remaining left ventricle was homogenized manually with a Dounce homogenizer (~20 strokes) in the presence of Proteinase K (0.8 mg in 5 ml of IB, P8038, Sigma). Homogenized tissue was centrifuged 5 min at 480*g* (4 °C), and the pellet containing unbroken cells and nuclei was discarded. The resulting supernatant containing the mitochondrial fraction was further centrifuged at 7700*g* (3 × 5 min), and the final pellet was resuspended in IB with no EGTA and further centrifuged at 7700*g* for 5 min. Protein concentration of the mitochondrial suspension was determined as described [52].

Subcellular fractionation

Subcellular fractionation was performed as described by Kumar et al. [33] with small modifications. Briefly, rat heart was removed and ventricles separated and washed in 20 ml of IB, as above. Ventricles were cut with scissors and permeabilized by resuspension in the following ice-cold buffer (in mmol/l): 210.0 sucrose, 20.0 HEPES/KOH (pH 7.5), 10.0 KCl, 1.5 $MgCl_2$, 1.0 EDTA, 1.0 EGTA, 1.0 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, protease inhibitor cocktail (1 MiniComplete Tablet, Roche), and 0.04% Triton X-100, and were fully homogenized. To sediment nuclei and debris, ventricular lysates were centrifuged at 600×*g* (2200 rpm, Sorval SS-34 rotor) for 10 min at 4 °C. The supernatant was further centrifuged at

12,000×*g* (10,000 rpm, Sorval SS-34 rotor) for 25 min at 4 °C. After this centrifugation, the pellet was used as mitochondrial fraction and the supernatant as a cytosolic fraction, and was used for immunoblot analysis.

Preparation of rat heart membranes

Freshly isolated rat ventricles were separated and homogenized with a Brinkmann Homogenizer (Brinkmann Instruments, Westbury, NY, USA) in 4 ml of ice-cold solution, containing 0.32 M sucrose, 1 mM EGTA, 0.1 mM EDTA, 10 mM Hepes, pH 7.5 and protease inhibitors (Complete Mini protease inhibitor cocktail tablets, Roche, Germany). Homogenates were centrifuged at 1440×*g* for 5 min in a Beckman G5-6K centrifuge. Supernatants were removed and centrifuged at 66,700×*g* for 30 min at 4 °C in a Beckman TLA 100.4 rotor. The resulting membrane fraction was resuspended in 300 µl of PBS containing (mM): NaCl 140, KCl 3, Na_2HPO_4 6.5, KH_2PO_4 1.5, and pH 7.5. Protein was quantified by Bradford assay, and 125 µg of protein used for immunoblots.

Protein expression

Expression construct for NBCe1 has been described previously [16]. Human embryonic kidney (HEK) 293 cells were grown at 37 °C in an air/CO_2 (19:1) environment in Dulbecco's modified Eagle's medium supplemented with 10% (v/v). HEK cells were transfected with NBCe1 cDNA, using the calcium phosphate method [45].

Measurement of mitochondrial pH

The protocol described by Kapus et al. was used with slight modifications [30]. Isolated mitochondria were suspended in a solution containing 40 mM sucrose, 5 mM Tris/Cl, 0.1% w/v albumin, 0.6 mM EGTA, to a final concentration of 40–50 mg/ml, and loaded with 10 µM BCECF-AM for 20 min at room temperature. BCECF-loaded mitochondria were then diluted 20-fold with the ice-cold final preparation medium containing 75 mM sucrose, 225 mM mannitol, 0.4 mM Tris/EGTA, 0.4 mM K^+-EGTA, 5 mM Tris/Cl, 0.1 mg albumin/ml, and pH 7.4, washed, and resuspended in the same solution in the presence of 10 mM NaCl for 4 min at room temperature. Mitochondria were then recentrifuged and resuspended to obtain the stock suspension (40–60 mg/ml). Mitochondria (0.8–1 mg) were suspended in 2 ml of basic medium containing 100 mM KCl, 80 mM sucrose, 10 mM K^+-Mops, 2.5 µg/ml oligomycin, 1 µM rotenone, and pH 7.0, and the suspension was added to a stirred cuvette maintained at 37 °C. BCECF fluorescence intensity was measured at excitation 503 and 440 nm, and emission collected at 535 nm, on an Aminco-Bowman series II (Silver Spring, Maryland, USA) spectrophotometer. Calibration of fluorescence in terms of matrix pH (pH_m) was carried out using the high K^+-nigericin method.

Measurement of mitochondrial Na^+/H^+ exchange activity

pH_m dependence of the Na^+/H^+ transport was measured by Na^+-induced changes in matrix pH. Briefly, after mitochondria were quickly exposed to NH$_4$Cl (10 mM, 4 min) to induce mitochondrial matrix acidification, mitochondria preparation was suspended in a MOPS-free basic medium. Following the spectrofluorometer-recorded fluorescence stable signal of over 1 min, 20 µl of 5 M NaCl was added (to a final of 50 mM Na^+) and the initial rate of the shift in pH was recorded. Other experiments were performed in the presence of the NHE1 Na^+/H^+ exchanger inhibitor, cariporide (HOE 642, 10 µM).

Mitochondrial swelling determination

Mitochondrial swelling was measured as a decrease in the 90° light-scattering signal induced by the addition of $CaCl_2$ to a final concentration of 200 µM, which promotes influx of solutes through the opened MPTP and decreases light scattering. After 5 min of preincubation at 37 °C in a medium containing (in mmol/l) 120 KCl, 20 MOPS, 10 Tris–HCl, and 5 KH_2PO_4 pH 7.4, and finally a $CaCl_2$ was added to a final concentration of 200 µM to induce MPTP opening [7, 39]. The decrease in light scattering was detected with a temperature-controlled Aminco Bowman Series 2 spectrofluorometer operating with continuous stirring at excitation and emission wavelengths of 520 nm. Light-scattering decrease was calculated for each sample as the difference between the values before and after the addition of $CaCl_2$. Experiments were performed in the absence and the presence of the NBC inhibitor S0859 (1 µM). The effect of the S0859 was expressed as the percentage of decrease in light scattering compared with that induced by 200 µM $CaCl_2$ (control).

Double immunostaining of rat cardiac myocytes and human embryonic kidney cells, and analysis by confocal microscopy

Freshly isolated adult rat cardiomyocytes were fixed onto 22 × 22-mm laminin (30 µg/ml)-coated glass coverslips, or HEK293 cells were fixed on glass cover slips and incubated at 37 °C for 30 min to allow attachment. Cells were rinsed with PBS and fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature. Myocytes were then washed with PBS and permeabilized with

0.1% Triton X-100 (v/v) in PBS for 15 min at 20 °C, and HEK 293 cells were washed with PBS and permeabilized with 0.1% Triton X-100 (vol/vol) in PBS for 2 min at RT°. After washing (2 × 5 min with PBS) and blocking (5% BSA in PBS, 20 min) were completed, myocytes or HEK cells were incubated with a combination of primary rabbit polyclonal anti-SLC4A7 antibody (NBC3, L15 sc-99633, Santa Cruz) and 200 nM MitoTracker Red CMXRos (M-7512, ThermoFisher Scientific), followed by secondary chicken anti-rabbit conjugated to Alexa fluor (1:100 dilution), or HEK cells were incubated with a combination of primary rabbit polyclonal anti-NBCe1 loop 4 antibody (1:500 dilutions) [16], and mouse anti-cytochrome *c* oxidase complex I (COXI) antibody (sc-58347, 1:2000; Santa Cruz) (1:100 dilutions), followed by secondary chicken anti-rabbit conjugated to Alexa fluor 488 and chicken anti-mouse conjugated to Alexa fluor 594 (1:100 dilutions). Immunostained cells were mounted in Prolong Gold antifade solution (P36935, ThermoFisher Scientific) and imaged with a Zeiss LSM-410 laser-scanning confocal microscope. Images were collected with an oil immersion 60× objective, confocal aperture 0.2, zoom 1.5× (numerical aperture 1.4, plan Apochromat) at a resolution of 0.5- to 0.7-μm field depth. Filtering was used to integrate the signal collected over four frames to decrease noise, with a frame size of 1024 × 1024 (scan time of 3.2 s/frame). Images were saved and visualized with the Fluoview 3.3 Software LSM Image Browser.

Colocalization analysis

For the colocalization assay of cultured HEK293 cells and isolated rat cardiomyocytes, images were obtained with the same settings of the confocal microscope, except for detector gain adjustments in the green channel that were performed to normalize saturations levels. Images were analyzed using the colocalization menu of the Image-Pro Plus software. Regions of interest (ROIs) covering the entire area of the cardiomyocyte were created, followed by the adjustment of the red-channel lower threshold to exclude background staining from further analysis. Colocalization statistics were determined from the points included within ROIs and presented as Pearson's Correlation values that can vary between 0 and 1. Pearson's correlation reflects the linear relationship between two variables. A correlation of zero means that there is no linear relationship between the variables, whereas a correlation of 1 indicates perfect positive linear relationship.

Immunodetection

Cell samples (50 μg protein), heart lysates (50 μg protein), or heart mitochondrial lysates (50 μg protein) were resolved by SDS-PAGE on 8–10% acrylamide gels, as indicated. Proteins were transferred to polyvinylidene fluoride membranes and then incubated with 1/1000 dilution of rabbit anti-NHE1 antibody (H-160, sc-28758, Santa Cruz), or 1:2000 dilution of mouse anti-COXI antibody (sc-58347, Santa Cruz), or 1:500 dilution of rabbit anti-SLC4A7 antibody (NBC3, L15 sc-99633, Santa Cruz), or 1:1000 rabbit anti-VDAC antibody, or 1:1000 rabbit anti-Na^+/K^+ ATPase antibody (H-300, sc-28800, Santa Cruz), or 1:1000 mouse anti-SERCA2 antibody (Thermo Scientific), or 1:1000 mouse anti-CAII antibody (H-70, sc-25596, Santa Cruz), or 1:200 mouse anti-caveolin-3 antibody (A3, sc-5310, Santa Cruz). Immunoblots were then incubated with 1:1000 dilution of donkey anti-rabbit IgG (sc-2317, Santa Cruz), or sheep anti-mouse IgG (NA931 V, Amersham Biosciences), or 1:2000 donkey anti-mouse. Blots were visualized with enhanced chemiluminescence reagent (ECL, Millipore) and a Chemidoc Station (Bio-Rad).

Tissue preparation, post embedding immunohistochemistry, and electron microscopy analysis

Isolated heart mitochondria were fixed for 2 h in 2% glutaraldehyde fixative. After fixation, tissue blocks were rinsed with PBS and post-fixed with 1% osmium tetroxide (2 h, 4 °C). Tissues were then dehydrated and included in Epoxi resin for 36 h subsequently at 35, 45, and 60 °C to allow polymerization. Ultra-thin sections (60 nm thick) were cut with a Reichert-J Super Nova microtome, and collected on copper grids. Samples were finally treated with lead citrate (1%) and uranyl acetate (1%) and viewed with a JEOL JEM 1200-EXII Electron Microscope. Micrograph images were captured and collected with an Erlangshen ES 1000 W digital camera.

Statistical analysis

All results are presented as mean ± SEM. Significance of differences between any two groups was determined by the Student's *t* test or ANOVA. A final value of $P < 0.05$ was considered significant for all analyses. All probability values reported are two-sided.

Results

Expression of the mitochondrial NHE1 Na^+/H^+ exchanger in the hypertrophic myocardium

Increased NHE1-mediated Na^+/H^+ exchange activity in the hypertrophic myocardium of SHR suggests

posttranslational regulation of the protein [10], without changes in the amount of NHE1 protein expressed in SHR hearts [48]. To determine the role of the mitochondrial NHE1 in mitochondria of hypertrophic hearts, we examined the expression of mitochondrial NHE1 in mitochondria isolated from adult SHR left ventricular myocardium and its respective age and sex-matched control (Wistar) rat hearts. Table 1 shows values of body weight (BW), heart weight (HW), right and left ventricular weights (RVW and LVW), and left ventricular mass index (LVMI, $LVMI = LVW \cdot BW^{-1}$), of 18- and 35-week-old hypertrophic (SHR) and normal (Wistar) rats. Hearts from SHR exhibited myocardial hypertrophy, as shown by the elevated HW, LVW, and increased LVMI compared to control, at both 18 and 35 weeks of age. Interestingly, cardiac hypertrophy seems to plateau in SHR at 18 weeks of age, as demonstrated by the LVMI values obtained at 18 and 35 weeks of age.

Mitochondria lysates were prepared from left ventricles from hypertrophic and normal rat hearts, and expression of NHE1 protein was examined by immunoblots. The purity of mitochondrial fractions obtained from rat left ventricles was assessed by electron microscopy. Electron micrographs showed well-preserved elliptical-shaped mitochondria with intact inner and external mitochondrial membranes, in mitochondria isolated from SHR and control rat hearts (Fig. 1). Interestingly, we noticed an increased number of enlarged mitochondria ("swollen") with evident irregularities of the cristae and increased number of disrupted mitochondria in 35-week-old SHR hearts, relative to Wistar mitochondria (*not shown*), as previously demonstrated in old failing SHR hearts [29]. NHE1 expression was analyzed in left ventricular mitochondrial lysates of Wistar and SHR rats by Western blot (Fig. 2). Cytochrome *c* oxidase subunit 1 (COX1) expression was used as a loading control (Fig. 2a). Expression of mitochondrial NHE1 was quantified by densitometry of the immunoblots and was corrected for loading differences by normalization to COX1. Mitochondrial NHE1 expression increased by ~40% in the hypertrophic myocardium of SHR compared to control rats, at 18 weeks of age (Fig. 2b). In addition, left ventricles of SHR showed ~50% increased expression of mitochondrial NHE1, compared to Wistar, at 35 weeks of age (Fig. 1b).

Na^+-induced changes in the matrix pH of isolated rat mitochondria

To determine if the increased expression of mitochondrial NHE1 in the hypertrophic myocardium of SHR correlates with increased transport activity of the exchanger, isolated mitochondria of SHR and control rats were loaded with BCECF-AM and the maximal rate of pH_m change measured after the addition of 50 mM NaCl to the cuvette. Previously, this experimental approach demonstrated that pH_m favours the entry of Na^+ via the Na^+/H^+ exchange transport pathway [30]. Herein, after achieving a steady-state value of pH_m and when the fluorescence signal was stable, 50 mM Na^+ was added and the shift in pH_m was recorded (Fig. 3). Heart mitochondria from 18-week-old SHR rats showed greater changes in pH_m compared to Wistar (Fig. 3a). Furthermore, at 35 weeks, mitochondria from the hypertrophic myocardium of SHR displayed a significant difference on NHE-driven maximal pH_m change compared to normal rats (Fig. 3a). Interestingly, Na^+/H^+ exchange activity was higher in SHR and Wistar rats at 35 weeks than at 18 weeks. The hypertrophic hearts presented ~50 and ~60% increase in maximal pH_m change upon the addition of Na^+, compared to normal hearts (Fig. 3b), measured at 18 and 35 weeks of age, respectively. To confirm that the Na^+/H^+ exchange activity measured in isolated rat heart mitochondria arose from NHE1 Na^+/H^+ exchanger, experiments were repeated in the presence of HOE to block NHE1. Indeed, 10 μM HOE inhibited mitochondrial NHE1 activity by ~80% (Fig. 3c).

Taken together, the differences in the Na^+/H^+ exchange activity observed in the myocardium of SHR rats compared to control rats matched the increased expression of the

Table 1 General characteristics of spontaneously hypertensive rats (SHR) and normal rats (Wistar)

Rat strain	Age (weeks)	Body weight (g)	Heart weight (g)	LVW (g)	RVW (g)	LVMI (mg)
Wistar (*n* 1)	18	334 ± 12	0.73 ± 0.02	0.55 ± 0.02	0.18 ± 0.01	1.64 ± 0.03
Wistar (*n*)	35	396 ± 13	0.90 ± 0.05	0.70 ± 0.05	0.20 ± 0.01	1.77 ± 0.15
SHR (*n*)	18	310 ± 18	0.97 ± 0.04*	0.78 ± 0.04*	0.22 ± 0.01	2.69 ± 0.05*
SHR (14)	35	381 ± 14	1.25 ± 0.02*	1.01 ± 0.02*	0.25 ± 0.01	2.74 ± 0.05*

Values are mean ± SEM

n number of animals, *BW* body weight, *HW* heart weight, *LVW* left ventricular weight, *RVW* right ventricular weight, *LVMI* left ventricular mass index

* $P < 0.05$ compared to age matched Wistar, *t* test

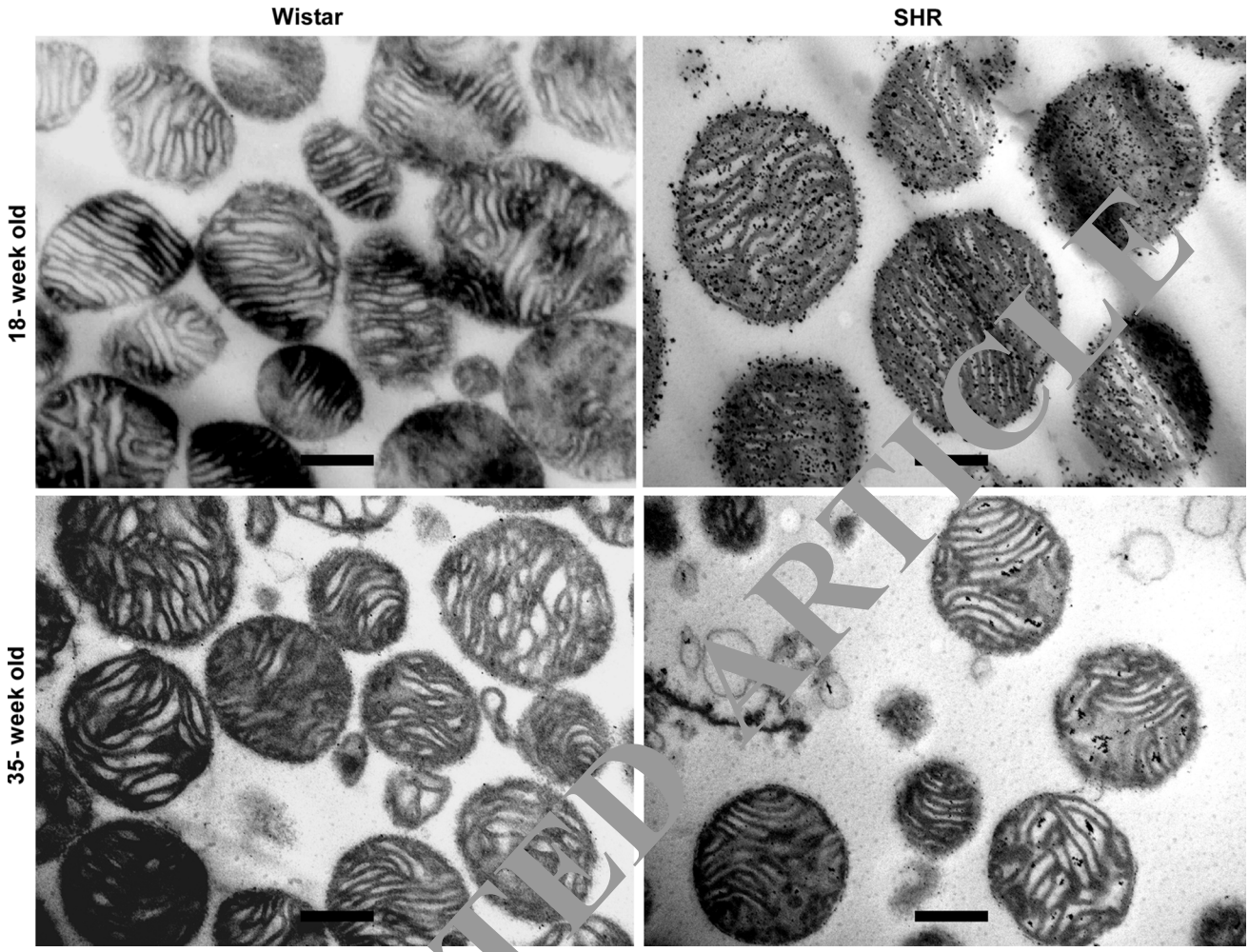

Fig. 1 Transmission electron microscopy images of isolated myocardial mitochondria. Images of left ventricular mitochondria, isolated from 18- and 35-week-old hypertrophic SHR and normal Wistar rat hearts, as indicated. Displayed ultra-thin sections were collected on copper grids and contrasted with lead citrate and uranyl acetate. *Scale bars* 0.5 μm

mitochondrial NHE1 Na^+/H^+ exchanger at both 18 and 35 weeks of age.

We recently reported that the MPTP opening and subsequent mitochondria volume increase triggered by Ca^{2+} arose, at least in part, from NHE1 activity [52]. Since NHE1 expression and function increase in the hypertrophic myocardium of SHR rats, we aimed to investigate the Ca^{2+}-induced MPTP opening and consequent mitochondrial swelling in isolated mitochondria of 18- and 35-week-old SHR and Wistar rats.

Mitochondrial swelling in mitochondria from hypertrophic and normal hearts

Measurements of Ca^{2+}-induced mitochondrial swelling were evaluated in energized mitochondria isolated from left ventricles of hypertrophic and normal rats, at 18 and 35 weeks of age (Fig. 4). Heart mitochondrial suspensions were exposed to 200 μM $CaCl_2$ to induce MPTP opening with the consequent mitochondrial swelling. These changes were observed as light scattering decreases (LSD) (decreased absorbance, Fig. 4a) after the addition of Ca^{2+}. Light-scattering decrease was larger in mitochondria from 35-week-old SHR and Wistar rats, compared to younger animals (Fig. 4a). Surprisingly, at 18 weeks of age, SHR rats showed little decrease in LS and a significant reduction in mitochondrial swelling, 67 ± 10% ($n = 15$ mitochondrial suspensions) vs. 100 ± 8% of control ($n = 13$ mitochondrial suspensions), measured as a percentage of compared LSD (Fig. 4b). Interestingly, hypertrophic SHR mitochondria displayed a slight reduction in threshold to MPTP opening and a moderate but not significant reduction on mitochondrial swelling, compared to normal, at 35 weeks of age ($n = 4$ mitochondrial suspensions) (Fig. 4b).

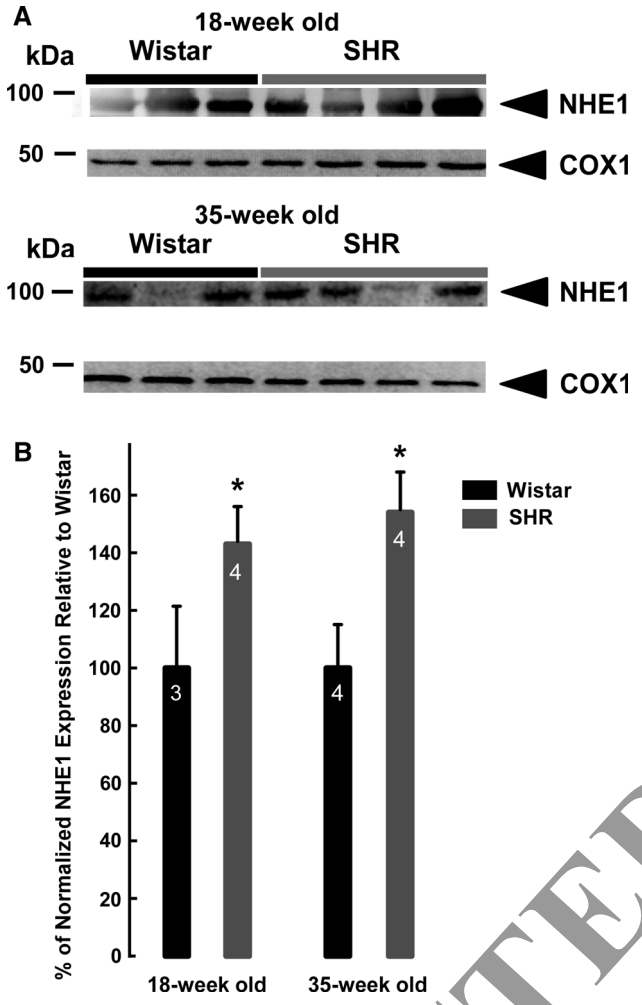

Fig. 2 Expression of mitochondrial NHE1 Na^+/H^+ exchanger in hypertrophic and normal rat heart mitochondria. **a** Rat heart mitochondrial lysates (100 μg) isolated from 18-week-old (*top*) and 35-week-old (*bottom*) SHR and Wistar rats analyzed by 7.5% SDS-PAGE, transferred to PVDF membranes, and probed with antibodies indicated at the bottom of each directed against NHE1, or COXI. **b** Average NHE1 protein expression, normalized to the amount of COX1. $*P < 0.05$. *Number on bar* indicates rat heart analyzed

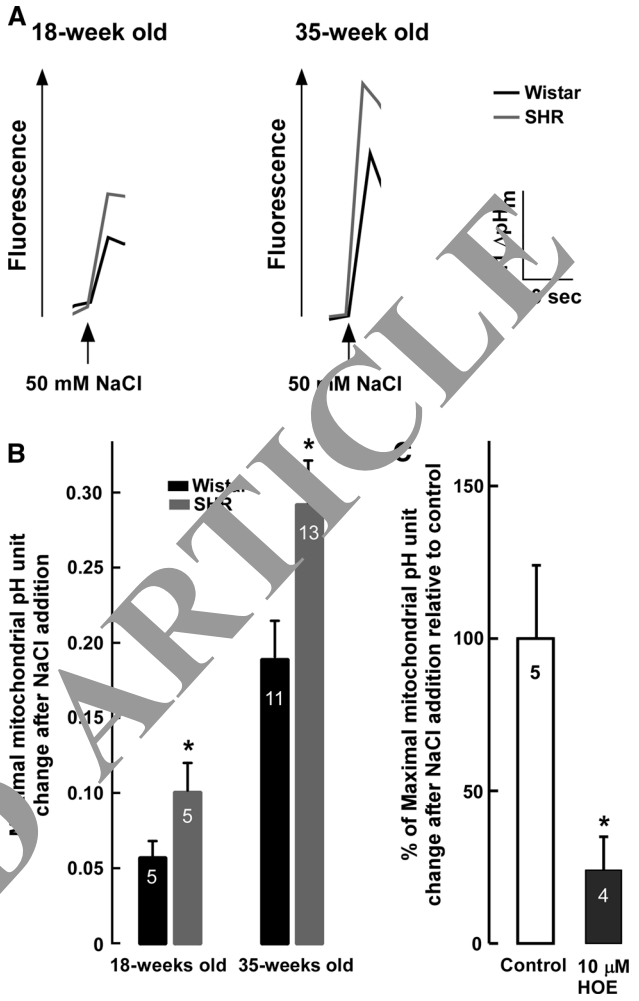

Fig. 3 Na^+-induced changes in matrix pH, in hypertrophic, and normal rat heart mitochondria. Freshly rat heart mitochondria (1 μg) isolated from 18- and 35-week-old SHR and Wistar rats were loaded with BCECF-AM and suspended in 2 ml of the basic medium. Where indicated 20 μl of 5 M NaCl (50 mM, final concentration) was added to the cuvette and changes in fluorescence were recorded. **a** Smooth curves of shift in pH after the addition of Na^+ (*arrows*), in non-respiring SHR (*gray*) and Wistar (*black*) rat heart mitochondria. **b** Maximal mitochondrial pH unit change was determined at the maximal point reach after Na^+ addition, in 18- and 35-week-old SHR and Wistar mitochondria. **c** Summary results of the % of pH shift after addition of Na^+ relative to control, in the presence or absence of 10 μM HOE. $*P < 0.05$. *Number* on *bar* indicates rat heart mitochondria analyzed

Effect of pharmacological inhibition of the mitochondrial NBC Na^+/HCO_3^- cotransporter on Ca^{2+}-induced mitochondrial swelling

Inhibition of NBC Na^+/HCO_3^- cotransport activity with the generic high-affinity potent S0859 compound, or targeted knockdown of SLC4A7 (NBCn1) demonstrated that the NBCn1 is co-localized with mitochondria, modulating the mitochondrial pathway of apoptosis during ischemic stress of culture coronary cells [33]. Here, isolated mitochondria from SHR and Wistar rat hearts were treated with 1 μM S0859 NBC inhibitor and exposed to Ca^{2+}-induced MPTP opening and subsequent mitochondrial swelling (Fig. 5). Remarkably, inhibition of NBC with S0859 increased the LS in hypertrophic and normal rat heart mitochondria (Fig. 5a). Thus, Ca^{2+}-induced mitochondrial swelling was significantly increased upon NBC blockade (Fig. 5b), suggesting mitochondrial action of the NBC Na^+/HCO_3^- cotransporter. Hence, NBC-mediated HCO_3^- transport seems to play a protective role in the rat heart mitochondria, preventing the mitochondrial swelling and further mitochondrial dysfunction in hypertrophic hearts.

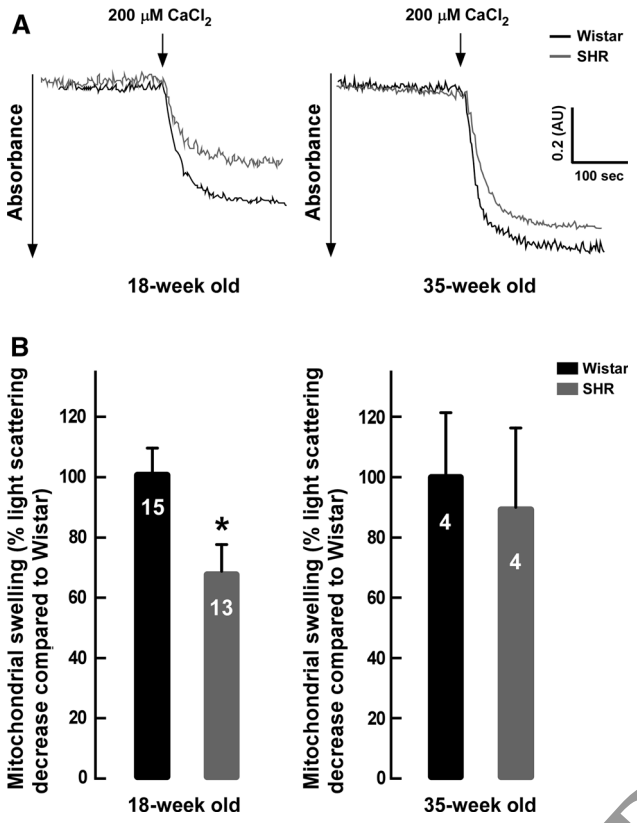

Fig. 4 Mitochondrial swelling induced by $CaCl_2$, in hypertrophic and normal rat heart mitochondria. **a** Sample scattered light absorbance decreases traces of Ca^{2+}-induced swelling in response to 200 μM $CaCl_2$ addition (*black arrow*), for heart mitochondria isolated from 18- and 35-week-old SHR (*gray traces*) and Wistar (*black traces*) rats. **b** Summary results of light-scattering decrease in hypertrophic (*gray bars*) and normal (*black bars*) rats, measured in mitochondria from 18- and 35-week-old age rats. $^{*}P < 0.05$ vs. Wistar. *Number* on *bar* indicates total experiments performed in each rat strain (4–15 heart analyzed)

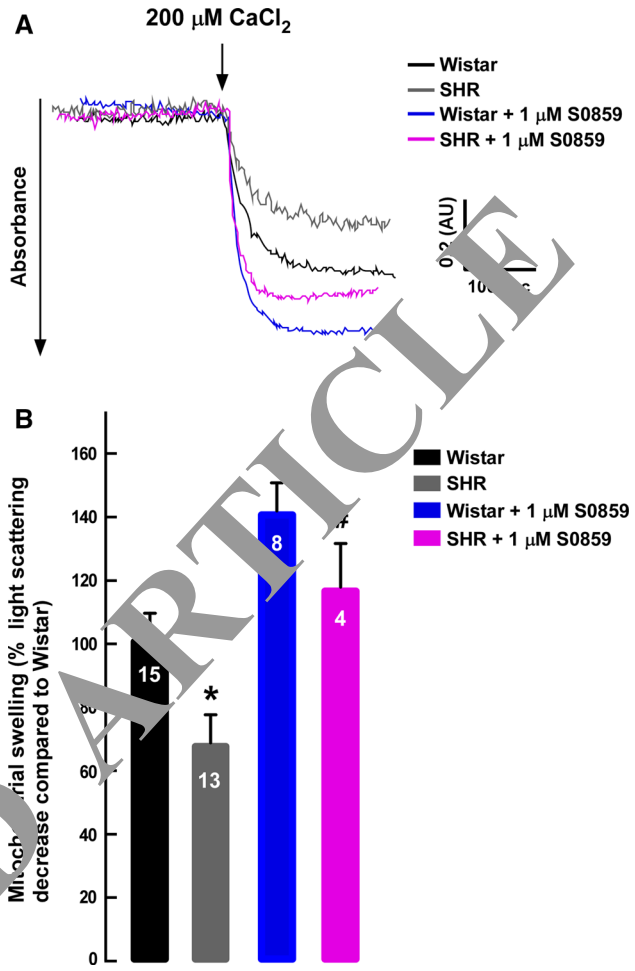

Fig. 5 Effect of the NBC inhibitor S0859 on Ca^{2+}-induced mitochondrial swelling, in hypertrophic and normal hearts. **a** Sample scattered light absorbance traces in the presence or absence of 1 μM S0859 after addition of $CaCl_2$, in 18-week-old (*pink*) and Wistar (*blue*) heart mitochondria. Traces of control experiments for SHR (*gray*) and Wistar (*black*) heart mitochondria were plotted considering original scales. **b** Summary results of percentage of light-scattering decrease in 18-week-old SHR and Wistar rat heart mitochondria, before and after treatment with 1 μM S0859. $^{*}P < 0.05$ vs. Wistar, and $^{\#}P < 0.05$ vs. SHR (ANOVA). *Number* on *bar* indicates total experiments performed in each rat strain (4–15 heart mitochondria sample analyzed)

Effect of pH on Ca^{2+}-induced MPTP susceptibility

Mitochondrial permeability transition pore opening is a key mechanism of injury in myocardial disease [56] and extracellular acidification decreases susceptibility to MPTP during early reperfusion [57]. Herein, we assessed the pH dependence of MPTP sensitivity on mitochondria exposed to different Ca^{2+} concentrations (Fig. 6). When experiments were performed at a normal 7.4 extracellular pH, isolated heart mitochondria exposed to 50 and 100 μM $CaCl_2$ elicited noticeable and transient LSD reminiscent of transient MPTP opening (Fig. 6a). However, when heart mitochondria were exposed to 200 μM $CaCl_2$ overload, a sustained and feasibly irreversible MPTP opening led to mitochondrial swelling (Fig. 6a, left panel, b). As previously reported [52], the LSD was attenuated by the NHE1 inhibitor HOE, at any given $[Ca^{2+}]$, consistent with a deleterious effect of NHE1 on Ca^{2+}-induced MPTP opening and consequent mitochondrial swelling (Fig. 6a, left panel, b). Conversely, S0859 promoted a further increase in LSD of rat heart mitochondria subjected to 50, 100, and 200 μM of $CaCl_2$ loading, supporting a protective role of the NBC cotransporter in mitochondria stressed with high $[Ca^{2+}]$ (Fig. 6a, left panel, b). In mitochondria maintained at acidic conditions (pH 6.8), however, Ca^{2+} loading stimulated significantly lower LSD compared to control pH. Interestingly, HOE did not prevent Ca^{2+}-induced LSD and mitochondrial swelling, in isolated heart mitochondria (Fig. 6a, right panel, b). S0859 did not affect LSD at 50 and 100 μM of $CaCl_2$ exposure, with small

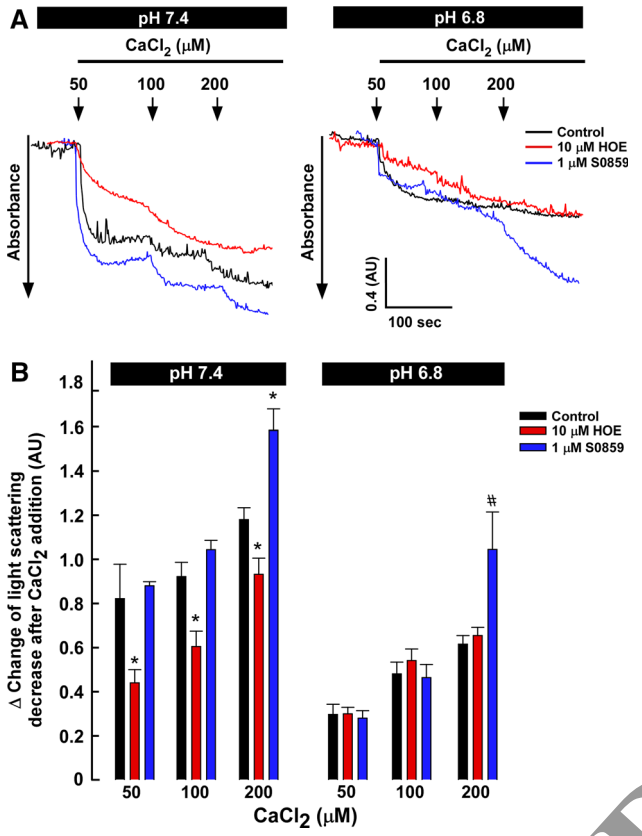

Fig. 6 Effect of pH on Ca^{2+}-induced MPTP susceptibility. Isolated heart mitochondria were pre-incubated by 10 min in the isolation media titrated to pH 7.4, or pH 6.8 and maintained throughout the experiments under these pH conditions. **a** Sample scattered light absorbance decrease traces of Ca^{2+}-induced swelling response to 50, 100, and 200 μM $CaCl_2$ addition (*black arrow*) for heart mitochondria maintained at control (7.40) or acidic (6.80) extracellular pH. **b** Summary results of Δ change of light-scattering decrease after $CaCl_2$ addition, in mitochondria maintained at pH 7.4 or maintained at pH 6.8. $^{*}P < 0.05$ vs. control (pH 7.4); $^{\#}P < 0.05$ vs. control (pH 6.8); *t* test. 4–7 heart mitochondria samples analyzed

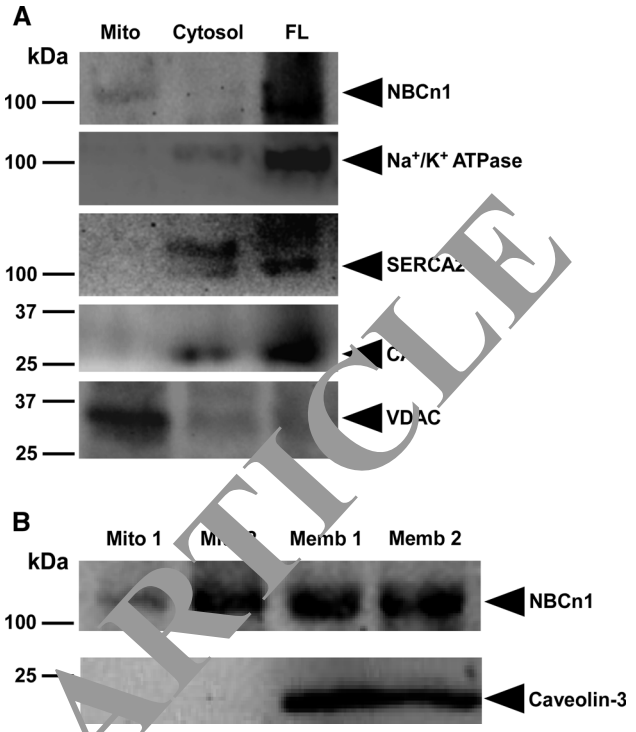

Fig. 7 Expression of the NBCn1 Na^{+}/HCO_3^{-} cotransporter in purified rat heart mitochondria and membrane lysates. Whole rat ventricular lysates were prepared by cellular and subcellular fractionation. **a** Western blot analysis of NBCn1, Na^{+}/K^{+} ATPase (sarcolemmal marker), SERCA2 (sarcoplasmic reticulum Ca^{2+} ATPase, sarcoplasmic reticulum marker), CAII (carbonic anhydrase isoform II, cytosolic marker), and VDAC (voltage-dependent anion channel, mitochondrial marker), using specific antibodies (*black arrowheads*). *FL* full lysate (50 μg protein), *Cytosol* cytosolic fraction (50 μg protein), *Mito* mitochondrial fraction (50 μg protein). Experiments were performed in duplicates. **b** Membrane lysates were prepared from rat heart ventricles. Protein samples (125 μg) were subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-NBCn1 (*top*) and anti-caveolin-3 (*bottom*) antibodies. *Filled arrow* indicates position of protein. Two separate mitochondrial and membrane fraction samples (1 and 2), corresponding to different hearts, were analyzed

effect on MPTP opening. Yet, when heart mitochondria were overloaded with high (200 μM) $[Ca^{2+}]$ in the presence of the NBC inhibitor S0859, LSD was markedly disturbed, consistent with a protective effect of NBC under acidic conditions (Fig. 6a, right panel, b).

Thus, acidic buffer protected heart mitochondria, indicated by decreased susceptibility to opening of the MPTP stressed with high $[Ca^{2+}]$. The NBC Na^{+}/HCO_3^{-} cotransporter seems to be a factor on this protection, particularly when mitochondria are overload with $[Ca^{2+}]$.

Expression of the NBCn1 Na^{+}/HCO_3^{-} cotransporter in mitochondria of hypertrophic hearts

The NBCn1 Na^{+}/HCO_3^{-} cotransporter is the most likely HCO_3^{-} transporter playing a functional role in heart mitochondria. We performed fractionation studies followed by verification with multiple marker proteins, to validate purity of mitochondrial fractions and corroborate expression of NBCn1 in rat ventricular muscle mitochondria and membranes. Immunoblot analysis of NBCn1 in ventricular lysates revealed a marked appearance of NBCn1 in the purified mitochondrial fraction (Fig. 7a). The purity of the mitochondrial fraction was confirmed by the absence of Na^{+}/K^{+} ATPase (sarcolemmal marker), SERCA2 (sarcoplasmic reticulum Ca^{2+} ATPase, sarcoplasmic reticulum marker), and carbonic anhydrase II (CAII, cytosolic marker), and the presence of VDAC (voltage-dependent anion channel and mitochondrial marker) (Fig. 7a). In addition, in a separate blot, the mitochondrial fraction was demonstrated to be negative for the specific heart muscle

plasmalemmal marker caveolin-3, a main component of the membranous subcompartment of the plasma membrane caveolae (Fig. 7b). Furthermore, relative expression of NBCn1 was demonstrated in purified membrane fractions of rat ventricles which were also positive for caveolin-3 (Fig. 7b). Therefore, we did not obtain contamination of subsarcolemmal mitochondria with plasma membrane or membrane-associated sarcoplasmic reticulum, demonstrating that besides ventricular sarcolemmal expression, there is a marked presence of NBCn1 in heart mitochondria.

On the basis of previous observations, NBCn1 is the candidate HCO_3^- transport system [33], playing a protective role in hypertrophic heart mitochondria exposed to high Ca^{2+} stress. To examine the role of the NBCn1 Na^+/HCO_3^- cotransporter in mitochondria of hypertrophic hearts, we tested the expression of NBCn1 in mitochondria isolated from SHR and control rat heart left ventricles (Fig. 8). Mitochondrial lysates were prepared, and expression of NBCn1 was quantified by densitometry of the immunoblots, corrected for loading differences by normalization to COX1 (Fig. 8a). Normalized expression of NBCn1 increased by ~90 and ~115% in the hypertrophic myocardium of SHR compared to Wistar, at 18 and 35 weeks of age, respectively (Fig. 8b).

We conclude that the NBCn1 Na^+/HCO_3^- cotransporter expression increased twofold in the hypertrophic heart, consistent with a protective effect.

Distribution of the NBCn1 Na^+/HCO_3^- cotransporter in isolated cardiomyocytes

To determine the cellular location of NBCn1 in the rat myocardium, fixed and permeabilized isolated adult rat cardiomyocytes were subjected to confocal immunofluorescence microscopy analysis using an isoform-specific rabbit polyclonal anti-NBCn1 antibody, which recognizes human, rat, and mouse NBCn1 (SLC4A7). Immunostaining of NBCn1 appeared in the sarcolemma and intercalated discs regions, with some transverse striated pattern suggestive of trafficking to the transverse *t*-tubules (Fig. 9), as previously shown [20]. Within the cell a longitudinal punctuate labeling of NBCn1, reminiscent of organelles, such as mitochondria, was observed. Therefore, we performed double labeling experiments of rat cardiomyocytes using MitoTracker red, a mitochondrial marker, and anti-NBCn1 antibody. Yellow/orange coloring in merged images demonstrates that NBCn1 colocalizes with MitoTracker red to some extent, suggesting expression of NBCn1 in cardiomyocyte mitochondria (Fig. 9). Additional dual-labeling experiments were carried out in HEK293 cells, which express endogenous NBCn1, to determine if NBCn1 is targeted to mitochondria as predicted. In HEK293 cell, the red fluorescence from the

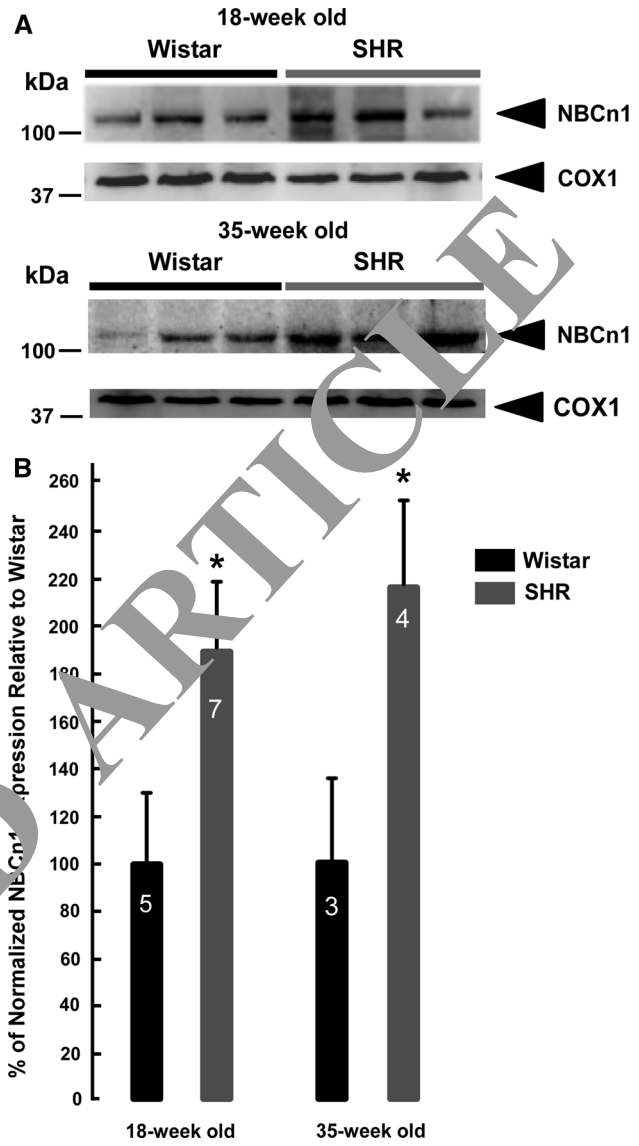

Fig. 8 Expression of mitochondrial NBCn1 Na^+/HCO_3^- cotransporter in hypertrophic and normal rat heart mitochondria. **a** Rat heart mitochondrial lysates (100 µg) isolated from 18-week-old (*top*) and 35-week-old (*bottom*) SHR and Wistar rats were analyzed by 7.5% SDS-PAGE, transferred to PVDF membranes, and probed with antibodies indicated at the *bottom* of each blot directed against NBCn1, or COXI. **b** Average NBCn1 protein expression, normalized to the amount of COX1. *$P < 0.05$. *Number* on *bar* indicates number of hearts isolated from SHR and Wistar rats and used for mitochondrial protein expression analysis

MitoTracker was found primarily in clusters of punctuate extranuclear sites, consistent with mitochondrial localization (*not shown*). Green fluorescence associated with NBCn1 is present at the cell surface and with a punctuate pattern in other subregions of the cell. These data suggest that, in addition to plasma membrane localization, NBCn1 is most likely targeted to the mitochondria of HEK293 cells (*data not shown*).

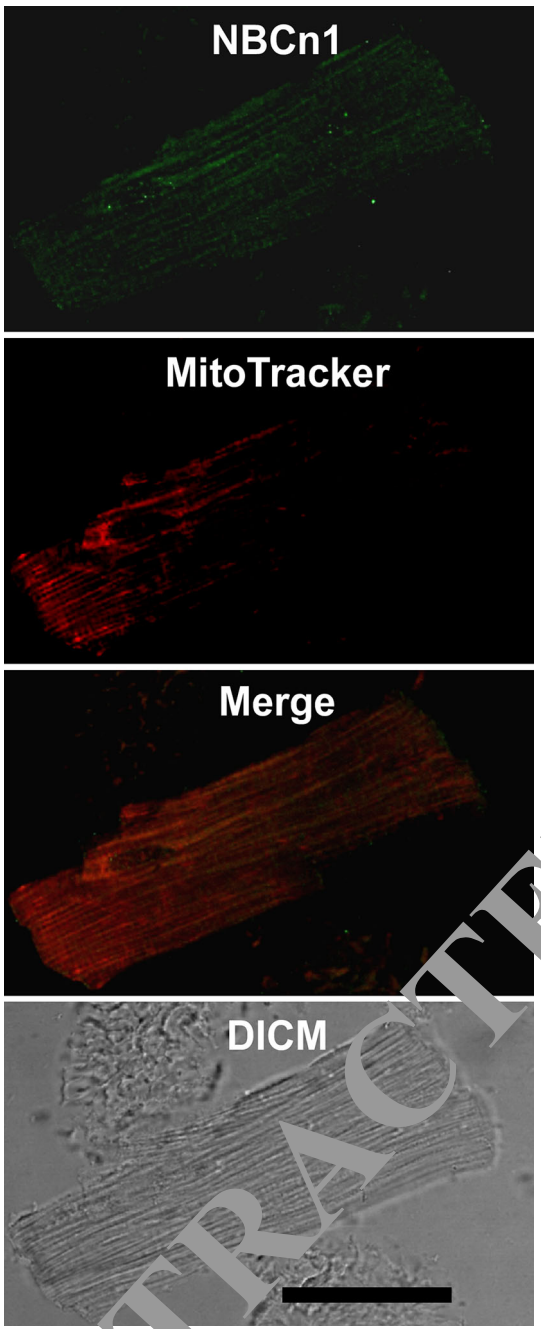

Fig. 9 Immunocytochemical analysis of the expression of NBCn1 in mitochon[dria] of rat cardiomyocytes. Freshly isolated adult rat cardiomyocy[tes] were double stained with rabbit anti-NBCn1 antibody or M[itoTracker] red as indicated and in green and red staining, r[espectively] followed by Alexa fluor 488-conjugated chicken anti-rab[bit] IgG. Co-localization of NBCn1 and MitoTracker is indicated as merge[d] with *yellow/orange* staining. *DICM* differential interference contrast microscopy. Images were collected with a Zeiss LSM-410 laser-scanning microscope. *Scale bars* 30 μm

We proposed here that increased expression of mitochondrial NBCn1 in hypertrophic heart has a protective role in mitochondria exposed to high Ca^{2+}. Following this line, we performed further immunofluorescence and confocal microscopy analysis of isolated normal and hypertrophic rat cardiomyocytes (Fig. 10). Merged images suggest extensive colocalization of NBCn1 and MitoTracker red in mitochondria of Wistar and SHR rat cardiomyocytes (Fig. 10a). The absence of fluorescence in cardiomyocytes treated with secondary antibodies alone indicates specificity of detection (Fig. 10b).

Quantification analysis was performed in isolated cardiomyocytes double-labeled with anti-NBCn1 antibody and MitoTracker red, using the "colocalization" function of the image analysis software Image-Pro Plus. The degree of association of NBCn1 with MitoTracker is depicted as a Pearson's Correlation coefficient (*r*) (Fig. 10c), where the *r* values between -0.3 ± 0 indicate little or no association, $+0.3 \pm 0.7$ weak positive association, and $+0.7 \pm 1.0$ strong positive association. NBCn1 shows a high degree of colocalization with MitoTracker in rat heart mitochondria (0.73 ± 0.04, $n = 32$), suggesting localization of NBCn1 to mitochondria. Interestingly, NBCn1/MitoTracker colocalization was higher in the hypertrophic myocardium, 0.80 ± 0.04 ($n = 10$), compared to normal, 0.70 ± 0.03 ($n = 16$) (Fig. 10c).

To examine the possibility of NBCe1 Na^{+}/HCO_3^{-} transporter expression in the heart mitochondria and to confirm that the NBCn1 is the sole NBC isoform involved in the prevention of the Ca^{2+}-induced MPTP opening, we performed additional immunocytochemistry and immunoblot experiments, using mitochondrial samples and isolated rat heart cardiomyocytes (Fig. 11). Immunoblot analysis of heart mitochondria revealed expression of NBCn1 (Fig. 11a, top). Conversely, antibodies against NBCe1 failed to detect NBCe1 in isolated heart mitochondria (Fig. 11a, bottom). Samples of HEK293 cells, or HEK293 cells transfected with NBCe1 cDNA were used as controls of NBCn1 (endogenously expressed) and NBCe1 (transiently expressed) expression, respectively (Fig. 11a, top and bottom). Furthermore, antibodies against NBCe1 recognize labeling consistent with sarcolemmal staining with invaginations that run toward the center of the myocytes, in isolated rat cardiomyocytes (Fig. 11b). Such a distribution is consistent with the presence of NBCe1 in the sarcolemma and along the transverse tubular system, as previously reported [16, 18, 20]. However, NBCe1 did not colocalize with the mitochondrial marker COX1 in isolated myocytes, using immunocytochemistry and combined confocal microscopy analysis (Fig. 11b).

We conclude that NBCn1 plays a functional role in heart mitochondria and that the prevention of the MPTP opening and subsequent mitochondria volume increase triggered by $CaCl_2$ arises, at least in part, from mitochondrial NBCn1.

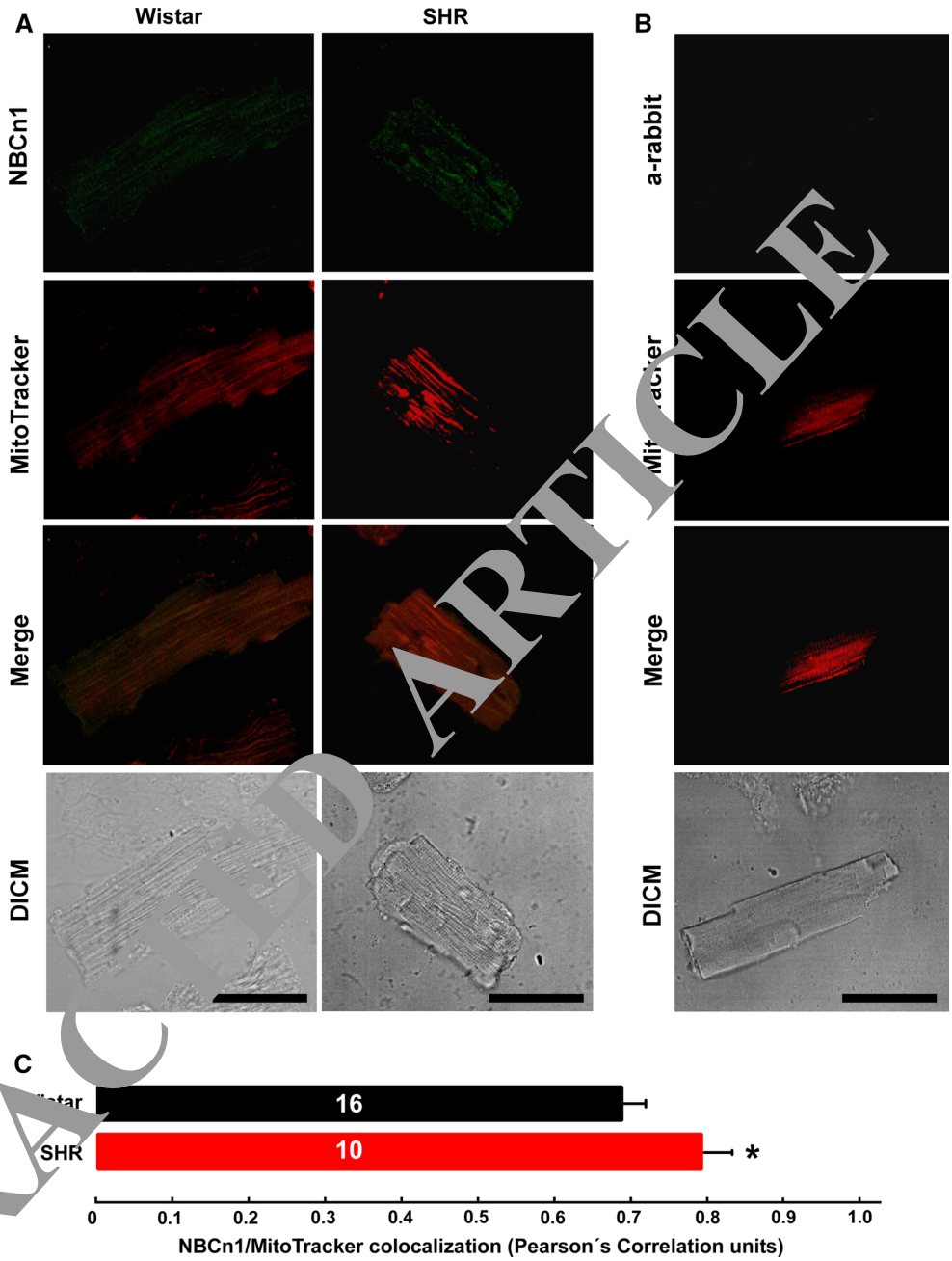

Fig. 10 Localization of NBCn1 in hypertrophic and normal rat cardiomyocyte. **a** Adult SHR and Wistar rat cardiomyocyte double stained with rabbit anti-NBCn1 antibody (*green*) and MitoTracker red, as indicated in the panels. Immunofluorescence signals were visualized by an Alexa fluor 488-conjugated anti-rabbit IgG antibody (*green*) and the fluorescence emitted at 594 nm. Images were collected with a Zeiss LSM 510 laser-scanning confocal microscope, with a ×63/1.4 oil immersion objective. Colocalization of NBCn1 and MitoTracker red is indicated as merge and with *yellow*/*orange* staining. **b** Isolated rat cardiomyocyte treated with secondary Alexa fluor 488-conjugated anti-rabbit IgG antibody and MitoTracker red. *DICM* differential interference contrast microscopy. *Scale bars* 30 µm. **c** Colocalization degree of NBCn1 and MitoTracker red in Wistar and SHR rats performed with the image analysis software Image-Pro Plus. Values represent Pearson's Correlation units (*r*) that reveal the degree of association of pixels of confocal images. Cardiomyocytes analyzed corresponds to three independent cell isolations. Number of cells analyzed is presented inside the *bars*. $^{*}P < 0.05$

Discussion

In the present study we characterized the role of mitochondrial NHE1 Na^+/H^+ exchanger and mitochondrial NBCn1 Na^+/HCO_3^- cotransporter in mitochondria of 18- and 35-week-old hypertrophic SHR rat hearts, in comparison with appropriate controls. The main findings were: (a) increased NHE1 expression and function in the myocardial mitochondria of SHR rats; (b) high expression of NBCn1 in rat heart; (c) colocalization of NBCn1 with mitochondria, and higher expression of NBCn1 in the mitochondria of hypertrophic hearts compared to normal; (d) increased mitochondrial NHE1 Na^+/H^+ exchange in the myocardium of SHR rats is compensated by enhanced mitochondrial NBCn1 activity, reducing Ca^{2+}-induced MPTP opening and mitochondrial swelling; (e) inhibition of NBCn1 aggravated mitochondrial MPTP opening and swelling in hypertrophic and normal rat heart mitochondria exposed to high Ca^{2+}; (f) acidic pH protected heart mitochondria by reducing MPTP opening in mitochondria exposed to high $[Ca^{2+}]$, and (g) NBC cotransporter inhibition exacerbates MPTP opening with subsequent mitochondrial swelling, playing a protective role under acidic conditions.

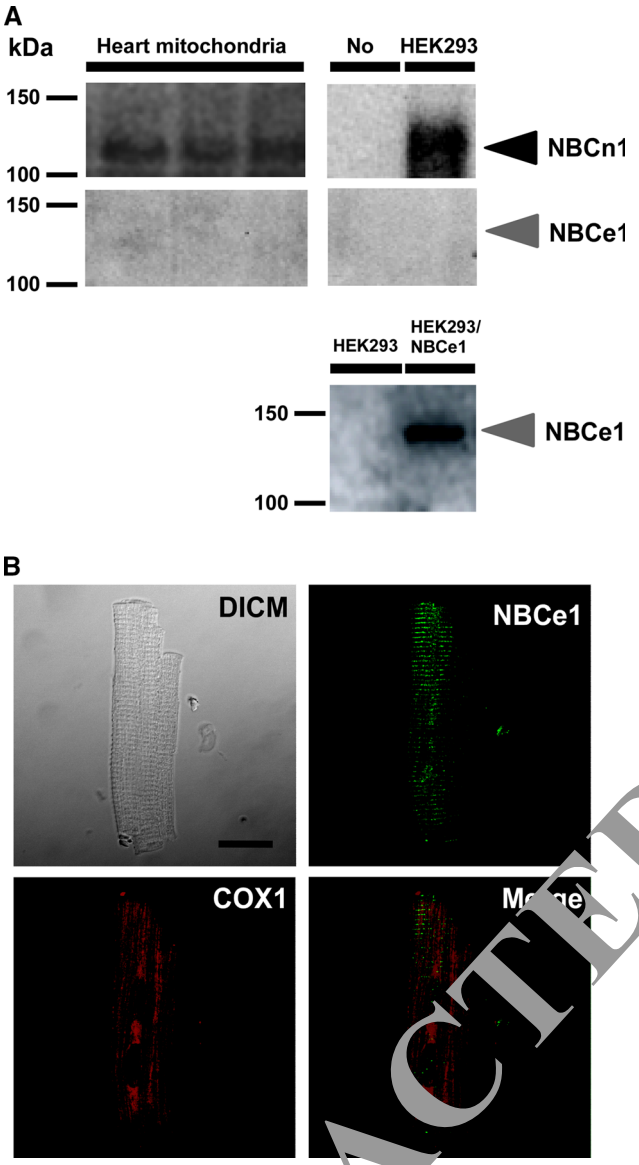

Fig. 11 Localization of NBCe1 in rat cardiomyocyte. **a** Rat heart mitochondrial lysates (100 µg) isolated from adult Wistar rats or lysates of HEK293 cells, or lysates of HEK293 cells transfected with NBCe1 cDNA, were analyzed by 7.5% SDS-PAGE transferred to PVDF membranes and probed with antibodies directed against NBCn1, or antibodies directed against NBCe1 (*black* and *gray arrows*, respectively). **b** Freshly isolated adult rat cardiomyocytes were double stained with rabbit anti-NBCe1 antibody or with mouse anti-cytochrome c oxidase complex I (COXI) antibody as indicated and in green and red staining, respectively, followed by Alexa fluor 488-conjugated chicken anti-rabbit IgG and Alexa fluor 596-conjugated chicken anti-mouse IgG. Colocalization of NHE1 and COX is indicated as a *merged* image and with *yellow* staining. Images were collected with a Zeiss LSM 510 laser-scanning confocal microscope, with a ×63/1.4 oil immersion objective. *Bar* 20 µm. *DICM* differential interference contrast microscopy

A large body of evidence supports the idea that mitochondrial dysfunction underlies cardiac diseases [6]. Under basic physiological conditions, mitochondria synthesize ATP to meet energy needs of the heart, and the Ca^{2+} signal is required to balance the rate of ATP production with the ATP demand in this normal context. When the heart is overloaded with Ca^{2+}, especially when this is accompanied by oxidative stress, mitochondria induce necrotic and/or apoptotic cell death. These latter events happen acutely in reperfusion injury and chronically in congestive heart failure [1]. Thus, Ca^{2+} overload, along with adenine nucleotide depletion and oxidative stress events, induces opening of a non-specific pore in the mitochondrial membrane, the MPTP. The MPTP, which opens transiently (physiological) or permanently (pathological), has been targeted as a mechanism to prevent reperfusion injury following an ischemic episode and to treat pathologic cardiac hypertrophy [23, 44]. In this line, delayed opening of MPTP by cyclosporin A protects from ischemia/reperfusion injury in perfused rat hearts [17, 22]. Moreover, cyclosporin A administered at the time of reperfusion decreased infarct size in humans with acute myocardial infarction [44]. Thus, MPTP inhibition is a key factor to prevent myocardial ischemia/reperfusion damage [24].

Interestingly, a role of mitochondria in determining cardiac hypertrophy after myocardial infarction has also been proposed [8, 9, 26, 28, 31]. As mentioned, MPTP opening can be further increased when Ca^{2+} overload is associated with oxidative stress. Moreover, two known enhancers of ROS production, angiotensin II (AngII), and endothelin 1 decreased mitochondrial anion superoxide formation following NHE1 inhibition [19]. Furthermore, the increase in ROS production induced by the opening of mitochondrial K_{ATP} was abolished by the NHE1 specific inhibitor HOE (cariporide) [19]. Electroneutral Na^+/H^+ exchange across the mitochondrial inner membrane contributes to organellar volume and Ca^{2+} homeostasis [21, 40]. The fluxes of Ca^{2+}, H^+, and Na^+ support a series of events in which the NHE has a central part [11]. As a consequence of its mitochondrial Na^+/H^+ exchange activity, NHE1 has a fundamental physiological/pathophysiological role in the mammalian heart [52]. Consistent with this, we found one major functional role for NHE1 in heart: the increase in threshold to open the MPTP in heart mitochondria deficient of NHE1, following post-transcriptional NHE1 gene silencing, or delay of MPTP opening upon selective blockade of mitochondrial NHE1 with HOE.

In addition to the complex interplay between the Na^+ transporters, channels, and exchangers on the sarcolemma, an important role for Na^+ transport across the mitochondrial inner membrane has been well recognized. Moreover, NHE1 is a key player influencing Na^+_i when ion homeostasis is disturbed under pathological conditions. Elevation of Na^+_i has been reported in both human and animal models of hypertrophy and heart failure, and NHE1 could

be responsible for the increased Na^+ influx into the heart (for review see O'Rourke and Maack [41]). In pacing-induced heart failing rabbits, NHE1 inhibition with BIIB722 (sabiporide) attenuated the functional, morphological, and biochemical derangements of the myocardial morphology, which includes the cardiac muscle mitochondria [3]. Furthermore, sabiporide reduced the high levels of circulating noradrenaline and improved the β-adrenergic receptors density and adenylyl cyclase activity, all factors altered in these failing rabbit hearts [37]. The increase in the $Na^+{}_i$ content detected in pathologic hearts would ultimately affect mitochondrial Ca^{2+} homeostasis. Thus, elevated $Na^+{}_i$ may impair mitochondrial Ca^{2+} loading and limit the stimulation of oxidative phosphorylation, increased dangerous O_2 radical mitochondrial production, and predispose the failing hearts to MPTP activation [41].

Extracellular acidification decreases cardiac injury during reperfusion in part via the transient and reversible inhibition of mitochondrial complex I, leading to a reduction of O_2 radical generation and accompanied by a decreased susceptibility to MPTP during early reperfusion [57]. At the onset of reperfusion, normalization of pH_i occurs with a concomitant intracellular Ca^{2+} accumulation and a burst of ROS generation that triggers MPTP opening [56]. Delayed pH_i recovery inhibits Na^+/H^+ exchange and results in less Ca^{2+} overload during reperfusion [25]. Thus, Ca^{2+} retention capacity of mitochondria was observed as reduced in ischemic hearts compared to non-ischemic hearts [57]. In the present manuscript, we showed that NHE-mediated Na^+/H^+ exchange activation had a damaging role in stressed mitochondria. Conversely, activation of the NBC Na^+/HCO_3^- cotransporter was protective at both, normal (7.40) and acid (6.8) pH, when mitochondria were exposed to high Ca^{2+}, resulting in permanent MPTP opening. Decreased Ca^{2+} overload during acidification achieved by the NBC may explain decreased MPTP susceptibility.

Electroneutral Na^+/HCO_3^- transport by NBCn1 was reported earlier in ventricular cardiomyocytes and Purkinje fibers [14, 35]. mRNA levels and activities of NBCn1 upregulate following pressure overload ventricular hypertrophy induced by aortic constriction [58]. In this context, enhanced NBCn1 activity in the course of cardiac hypertrophy progression [58], or during AngII stimulation [15] is expected to cause intracellular Na^+ overload, which would be detrimental to myocytes during ischemia/reperfusion due to Ca^{2+} overload via Na^+/Ca^{2+} exchangers. Recently, AngII-mediated alteration of cardiac NBC expression and function, which results in a decrease of the electrogenic NBCe1 at the cell surface and in a compensatory upregulation of the electroneutral NBCn1, was demonstrated in the hypertrophic myocardium of SHR [42]. In addition, the AT1 AngII-receptor antagonist losartan reversed these alterations, establishing that AngII is directly involved in the remodeling of NBC isoforms during the cardiac hypertrophy of SHR rat [42].

Lately, mitochondrial NBCn1 was found to modulate the mitochondrial pathway of apoptosis during ischemic stress in coronary cells [33]. Contrasting with the role of the mitochondrial NHE1, mitochondrial NBCn1 at the mitochondria has a protective role for the organelle and for the cardiac muscle cells subjected to pathologic stressors. How mitochondrial NBCn1 suppresses the mitochondrial apoptotic pathway under ischemic stress, however, remains unresolved. Previous reports suggested that ischemia-induced cytosolic acidification results in acidification of mitochondrial matrix, contributing to mitochondrial swelling [46], which may provoke cytochrome *c* release as an early event in the apoptotic cascade. Thus, NBCn1 could alkalinize the mitochondrial matrix, protecting the mitochondria from permanent MPTP opening, swelling, and subsequent apoptosis.

We propose that mitochondrial NBCn1 catalyzes HCO_3^- fluxes which regulate mitochondrial matrix pH homeostasis, influencing mitochondrial metabolism and MPTP opening. Increased NBCn1 expression and activity in mitochondria of hypertrophic hearts balanced the deleterious upregulation of NHE1 in SHR heart mitochondria, slowing the MPTP opening after Ca^{2+} overload. Permeability of the mitochondrial membrane to HCO_3^- ions has been discussed earlier, and the existence of transport mechanisms was suggested [47]. However, it is still unknown whether an HCO_3^- transporter regulates mitochondrial matrix pH under ischemic stress or under pathologic hypertrophic growth of the heart. We hypothesize that intra-mitochondrial Na^+-driven NBCn1-dependent HCO_3^- influx reduces matrix acidification, thus preventing mitochondrial swelling. Therefore, an HCO_3^- transporter present in the heart inner mitochondrial membrane may provide a mechanism to regulate substrate metabolism in response to acid–base changes, or may reduce oscillations in matrix pH when modifications in cytoplasmic pH occur [49]. In addition, the influx of HCO_3^- into mitochondria may lead to the release of CO_2 as a result of the CA-mediated HCO_3^- degradation, through the mitochondrial CAV enzyme. Thus, CO_2 produced in a "metabolon-like" fashion could serve as a potent scavenger of high levels of harmful ROS, as it has been proposed [51], maintaining mitochondrial integrity.

In addition to its protective role on modulating oxidative stress and apoptosis, NBCn1-mediated HCO_3^- influx into mitochondria activate HCO_3^- sensitive targets, such as soluble adenylyl cyclase (sAC) [13]. sAC is particularly activated and regulated by HCO_3^- ions and localized to mitochondria, producing cAMP in the organelle, to regulate oxidative phosphorylation [2]. Thus, sAC modulates

metabolic processes, including cytochrome *c* oxidase and the O_2 radical production, through the intra-mitochondrial cAMP/protein kinase A pathway [2]. Reduction of NBCn1-driven HCO_3^- influx may suppress mitochondrial sAC activity followed by an increase in mitochondrial ROS production, triggering apoptosis. In addition, following stimulation with HCO_3^- and Ca^{2+}, sAC produces cAMP, which in turn stimulates O_2 consumption, increases the mitochondrial membrane potential ($\Delta\Psi m$), and raises ATP production, representing a protective role for sAC against cell death, apoptosis, and necrosis in cardiomyocytes [54]. Furthermore, cAMP is rate limiting for matrix Ca^{2+} entry via the exchange protein directly activated by cAMP (Epac1) and the MCU mitochondrial Ca^{2+} uniporter. Together, this prevents harmful mitochondrial MPT opening [54].

Conclusions

We report the presence of the NBCn1 $Na^{+/}HCO_3^-$ cotransporter in rat heart mitochondria. We demonstrate a delay of the MPTP formation in isolated mitochondria of hypertrophic hearts exposed to Ca^{2+} overload, by NBCn1 activation. In addition, we found a protection in preservation of MPTP opening presumable due to less Ca^{2+} accumulation, in a mechanism that involves activation of the NBCn1 $Na^{+/}HCO_3^-$ cotransporter. This protection occurs when NBC was activated by hypertrophic (SHR) or acidic (6.7) pH stimuli, in heart mitochondria exposed to high $[Ca^{2+}]$.

These findings open a new avenue of research about the mechanism of protection achieved by activation of the mitochondrial NBCn1 HCO_3^--dependent pathway, either in reperfusion injury or in cardiac hypertrophy and failure.

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Compliance with ethical standards

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Conflict of interest None.

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